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(54) Title: A NOVEL POLYNUCLEOTIDE INVOLVED IN HERITABLE PARKINSON'S DISEASE

(57) Abstract: The invention relates to a polynucleotide encoding a leucine - rich repeat kinase 2 having a mutation in amino acid position 2019 (nucleotide position 6055). The polynucleotide consists of the base sequence of SEQ. ID. NO. 2, or a complementary strand thereto, wherein X is one of the group being defined by the bases A, C or T. The invention also relates to a primer and a probe specific for the polynucleotide, wherein the primer and/or probe contains at the least 10 consecutive nucleotides, and finally the use of the probe for proving parkinsonism inheritance.

A novel polynucleotide involved in heritable Parkinson's disease

Present invention relates to a novel polynucleotide involved in heritable Parkinson's disease (PD), a novel polypeptide encoded by the polynucleotide, and a method for diagnosing heritable Parkinson's disease (PD).

Background

Parkinsonism (MIM168600) is a clinical syndrome characterized by bradykinesia, resting tremor, muscle rigidity, and postural instability (Gelb et al. 1999). The most common cause of parkinsonism is Parkinson's disease (PD). Second to Alzheimer's disease, PD is the most common neurodegenerative disorder affecting >1% of the population over 55 years of age (de Rijk et al. 1995). Neuropathological findings in PD are loss of pigmented neurons in the brainstem, *substantia nigra* and *locus ceruleus*, with intracellular Lewy body inclusions found within surviving neurons (Forno 1996).

Although PD is considered a sporadic disease, various hereditary forms of parkinsonism have been recognized (Vila and Przedborski 2004). A major breakthrough in recent years has been the mapping and cloning of a number of genes causing monogenic forms of parkinsonism. Genomic multiplication and missense mutations in the α-synuclein gene were initially identified in a small number of families with autosomal dominant parkinsonism (PARK1/4 [MIM 168601]) (Polymeropoulos et al. 1997; Kruger et al. 1998; Singleton et al. 2003; Chartier-Harlin et al. 2004; Farrer et al. 2004; Zarranz et al. 2004). Subsequently, α-synuclein antibodies were found to robustly stain Lewy bodies and Lewy neurites in the *substantia nigra* in familial and sporadic PD (Spillantini et al. 1997) and common genetic variability in the α-synuclein promoter has been implicated in sporadic PD (Pals et al. 2004).

Autosomal recessive mutations in three genes, *parkin*, *DJ-1* and *PINK1* have been linked with early-onset parkinsonism (<45 years at onset) (PARK2, PARK6 & PARK7 [MIM 602533, 602544 & 608309])(Kitada et al. 1998; Bonifati et al. 2003; Valente et al. 2004). A large number of pathogenic mutations and rearrangements have been identified in the *parkin* gene reviewed by (Mata et al. 2004), but mutations in DJ-1 and PINK-1 are rare (unpublished data).

Very recently, five pathogenic mutations were identified in a gene, leucine-rich repeat kinase 2 (*LRRK2*) in six families with autosomal-dominant parkinsonism, linked to the PARK8 locus [MIM 607060]) (Zimprich et al. 2004a). Paisan-Ruiz and colleagues

independently confirmed these findings of two pathogenic mutations in a British and Basque families (Paisan-Ruiz et al. x2004).

Object

The object of the invention is to isolate a gene or polynucleotide proving inheritable parkinsonism, and to use presence of this gene to diagnose a patient before he/her gets sick. A further object is to use this gene or polynucleotide to transfect a microorganism or experimental animal in order to develop a new medicine for treating or preventing the onset of parkinsonism.

The invention

Inheritable parkinsonism may be proved by the method according to the characterizing part of claim 5, and the other objects are met by a polynucleotid according to the characterizing part of claim 1, a recombinant vector according to claim 3, a DNA probe and a DNA primer according to claims 4 and 6 respectively, and a peptide according to claim 9.

The inventors have isolated a novel LRRK2 mutation, and this mutation may cause development of dominantly inherited PD. By screening healthy persons, one can state whether the healthy persons have the mutation, and thus most likely will develop the illness.

Using a probe to test whether a patient has the mutation allows a precise, differential diagnosis of this type of Parkinson's disease. The probe represents a safe and accurate biomarker which will be powerful as it nominates subjects, future patients, for neuroprotective therapy. At the present time this is a research enterprise, but not for long. These subjects provide the first (and only) 'uniform substrate/background' for studies on drug efficacy/safety. From a research perspective **they** will also facilitate models of disease (C.elegans, Drosophila, mice) and epidemiological research on the variable expressivity and age-associated penetrance. **As the sequence of the mutated gene is known, microorganisms and further experimental animals may be transfected, in order to investigate for a new medicine to treat or prevent the onset of the illness.**

The genetic information provides subjects with the cause of their disease, an explanation for which, if handled correctly, can be of great psychological benefit (fulfilling the 'need to know' why). This information also prioritizes the resources of the research community,

grant funding agencies and the pharmaceutical industry on developing a neuroprotective therapies to halt G2019S disease progression.

In the following the invention will be described by reference to a study of PD patients and their families. Parts of the study are shown in figures, wherein

Figure 1 shows a schematic drawing of LRRK2 with predicted protein domains,

Figure 2 shows pedigrees of families with LRRK2 G2019S,

Figure 3 shows chromosome 12q12 STR markers on the disease haplotype (PARK 8),

Figure 4 shows probability of becoming affected by parkinsonism, in LRRK2 G2019S carriers, as a function of age, and

Figure 5 shows aligned amino acid sequences of the activation loop of different human kinases.

The inventors identified seven unrelated persons all having the new mutation, from 248 multiplex kindreds with dominantly inherited PD, and six further unrelated persons from three populaton-based series of persons with dominantly inhereted PD. These 13 persons and their families made basis for the inventors' further work. Segregation and linkage analysis provides evidence for pathogenicity and an estimate of age-associated penetrance; haplotype analysis demonstrates the mutation originates from a common and ancient founder.

Subjects and Methods

Study subjects

The patients and controls were examined by neurologists specialized in movement disorders. A full history, including family history and neurological examination, was completed on each patient. Clinical diagnosis of PD required the presence of at least two of three cardinal signs (resting tremor, bradykinesia and rigidity), improvement from adequate dopaminergic therapy and the absence of atypical features or other causes of parkinsonism.

LRRK2 sequencing and mutation screening

Blood samples were taken and genomic DNA was extracted using standard techniques. Six families (families 194, 281, 3081, 3082, 3083 and 3211) were known to have a positive LOD-score for STR (Short Tandem Repeat) markers in the PARK8 locus (Zimprich et al.

2004b). Amplification of all 51 exons of the *LRRK2* gene was performed by polymerase chain reaction (PCR) in one patient having PD, from each of these six families. All PCRs were carried out for each primer set with 20-50 ng of template DNA in a total volume of 25µl using a final reaction concentration of 200 µM dNTP, 1x PCR-Buffer (Qiagen), 1x Q-Solution (Qiagen), and 0.8 µM of each primer. One unit of Taq polymerase (Qiagen) was added to each reaction. Amplification was performed using a 57-52°C-touchdown protocol over 38 cycles. The primers used for PCR amplification of *LRRK2* exons and for sequencing are available on request.

The nucleotide sequences of all PCR products were determined by direct sequencing. Each PCR product was cleaned by using a Millipore PCR purification plate. Three microliters of purified PCR product was used per sequencing reaction with 1 µl of either the forward or reverse PCR primer and 1 µl of BigDye reaction mix (Applied Biosystems). Electrophoresis was performed under standard conditions on an ABI 3730 automated sequencer (Applied Biosystems). All sequences were obtained with both forward and reverse primers. Sequences were analyzed with SeqScape software version 2.1.1 (Applied Biosystems) and compared with published sequence of *LRRK2* (GenBank accession no. AY792511).

After identification of a heterozygous G2019S (G6055A) mutation in the proband of family 3215 (referred to as family 3211 in Zimprich et al, 2004b), we designed a probe employing TaqMan chemistry on an ABI7900 (Applied Biosystems) to screen for this mutation. First we examined 248 PD patients from families with a known family history, consistent with autosomal dominant transmission of a suspected causative gene. Then 377 Norwegian, 271 Irish and 100 Polish PD patients (constituting the three population series) were checked using this assay; and 2260 samples of healthy persons from similar populations were also included (1200 US American, 550 Norwegian, 330 Irish and 180 Polish subjects), the latter to be used as control samples. Mutations were confirmed by direct sequencing of PCR products from *LRRK2* exon 41. Finally, all participating family members of *LRRK2* G2019 mutation carriers (affected and unaffected) were screened for the mutation.

By 6055 G>A or G6055A it is meant that nucleotide number 6055 of the LRRK2 gene, counted from the 5'end of the polynucleotide, has changed from G (guanine) to A (adenine). This change also causes a change in the polypeptide encoded by the polynucleotide, and G2019S denotes a polynucleotide where aminoacid number 2019 is

changed from G(Glycine) to S(Serine). These shortenings are wellknown to persons skilled of the art.

Genotyping of STR markers

Fourteen STR markers were genotyped in mutation carriers and all available family members, in all 13 families, for linkage analyses and to determine whether there was a particular haplotype associated with the *LRRK2* mutation. STR markers were chosen to span the PARK8 region including D12S87, D12S1648, D12S2080, D12S2194, D12S1048, D12S1301 and D12S1701. *LRRK2* is located between D12S2194 and D12S1048. We also developed seven novel STR markers in this region (shown in table 1 below) by searching for repeat polymorphisms using RepeatMasker of *in silico* BAC sequence (UCSC Human Genome Browser Web site). The labeling of these novel markers reflects their physical position relative to the start codon of *LRRK2*.

Table 1. Novel chromosome 12 STR markers

Marker name	Primer sequence	Physical position (bp) On chromosome 12
D12S2514	F: 5'-TTGCAGCTGTAAGGAATTTGGG-3'	38873779
	R: 5'-GCATTCTTCAGCCTGAGACCC-3'	
D12S2515	F: 5'-TGAAGGACACTGAACAAGATGG-3'	38974140
	R: 5'-GCCATAGTCCTTCCATAGTTCC-3'	
D12S2516	F: 5'-CGCAGCGAGCATTGTACC-3'	38989214
	R: 5'-CTCGGAAAGTTTCCCAATTC-3'	
D12S2518	F: 5'-CTGGTATTACCTCAACTGTGGCTC-3'	39034800
	R: 5'-ACTGGTATGTTTAAGCCTGGCAC-3'	
D12S2519	F: 5'-AGCAGCAGAGAAGATTTCAATAAC-3'	39116816
	R: 5'-AATCATCTTTGAAAGAACCAGG-3'	
D12S2523	F: 5'-TAAACGAAGCTCCCTCACTGTAAG-3'	39147728
	R: 5'-TCTTTGTAGCTGCGGTTGTTTC-3'	
D12S2517	F: 5'-TCATGAAGATGTCTGTGATAGGGC-3'	39282976
	R: 5'-CTCTATTGTGAGCAAACTGCATGG-3'	

One primer of each pair was labeled with a fluorescent tag. PCR reactions were carried out on 10-20 ng of DNA in a total volume of 15 µl with final reaction concentrations of 150 µM dNTP, 1x PCR-Buffer (Qiagen), 1x Q-Solution (Qiagen) and 0.6 µM of each primer, with 1 unit of Taq Polymerase (Qiagen). Amplification was performed using a 57-52°C-touchdown protocol over 38 cycles. The PCR product for each marker was diluted by a factor of 10 to 100 with water. One microliter was then added to 10 µl of Hi-Di Formamide and Rox size standard. All samples were run on an ABI 3100 genetic analyzer, and results were analyzed using Genescan 3.7 and Genotyper 3.7 software (Applied Biosystems). Since population allele frequencies were not available from the CEPH database, these have been estimated by genotyping 95 unrelated Caucasian subjects, a population based series from the United States (shown in table 2 below).

Table 2. Allele frequencies of Park 8 Markers

Marker and allele (bp)	Frequency (%)
D12S87 (n = 92)	
150	0.5
154	1.1
156	27.2
158	33.2
160	11.4
162	2.7
164	6.0
166	17.4
168	0.5
D12S1648 (n = 91)	
110	13.7
112	3.3
114	11.0
116	4.4
118	2.2
120	2.8
122	17.0
124	3.9
126	7.7
128	14.3
130	8.8
132	2.8
134	2.8
136	1.7
138	0.6
140	2.2
142	1.1
D12S2080 (n = 93)	

Table 2. Allele frequencies of Park 8 Markers	
Marker and allele (bp)	Frequency (%)
176	1.6
180	20.2
184	44.7
188	22.9
192	10.6
D12S2194 (n = 87)	
245	0.6
249	40.9
253	32.4
257	19.9
261	4.6
265	1.7
D12S2514 (n = 82)	
284	11.0
291	53.1
294	32.3
297	1.2
300	2.4
D12S2515 (n = 93)	
208	3.2
212	26.6
216	18.6
220	22.9
224	20.7
228	5.3
232	2.7
rs 7966550 (n = 90)	
T	90.6
C	9.4
DS12S2516	
252	37.3
254	62.7
rs 1427263 (n = 89)	
A	63.6
C	36.5
rs1116013 (n = 88)	
A	49.4
G	50.6
rs11564148 (n = 88)	
A	26.1
T	73.9
D12S2518 (N = 90)	
154	79.7
168	15.9
170	4.4
D12S519 (n = 72)	
132	29.5

Table 2. Allele frequencies of Park 8 Markers	
Marker and allele (bp)	Frequency (%)
134	22.6
138	22.6
140	25.3
D12S2520 (N =85)	
248	8.2
251	7.6
254	10.0
257	54.1
260	20.0
D12S2521 (N = 93)	
311	0.5
315	10.8
319	20.4
323	8.1
327	7.0
331	8.1
335	0.5
355	1.1
359	7.5
363	13.4
367	7.0
371	7.0
375	6.5
379	3.8
383	1.1
387	.5
D12S2522 (N = 93)	
281	9.1
283	14.0
285	.5
287	11.3
293	.5
295	15.6
297	44.6
299	4.3
D12S2523 (n = 89)	
305	18.9
314	41.1
317	8.9
320	30.0
323	1.1

Table 2. Allele frequencies of Park 8 Markers

Marker and allele (bp)	Frequency (%)
D12S2517 (n = 93)	
180	8.5
182	7.5
184	15.4
186	8.5
188	11.7
190	8.0
192	5.3
194	1.1
196	1.1
198	3.2
200	0.5
202	3.7
204	6.9
206	6.9
208	4.3
210	2.1
212	3.2
214	1.6
216	0.5
D12S1048 (n = 89)	
211	37.2
214	21.1
217	17.8
220	2.2
223	6.7
226	11.7
229	3.3
D12S1301 (n = 93)	
96	0.5
100	37.2
104	17.6
108	11.1
112	12.2
116	13.3
120	7.5
124	0.5

Table 2. Allele frequencies of Park 8 Markers	
Marker and allele (bp)	Frequency (%)
D12S1701 (n = 93)	
89	4.3
91	4.8
93	10.8
95	40.0
97	16.0
99	12.4
101	11.8
103	0.5

A The number of individuals genotyped is given for each marker (n)
B Alle frequencies are for individual markers in U.S. control subjects

Statistical Analysis

Multipoint nonparametric LOD scores for all families were calculated using GENEHUNTER-PLUS (Kong and Cox 1997). The frequency of the deleterious allele was set at 0.0001, and empirically determined allele frequencies were employed. The map positions for each marker were taken from Rutgers combined linkage-physical map version 1.0 (MAP-O-MAT web site). The three loci D12S2080, D12S2194 and D12S1301 are very tightly linked, with no observed recombinants in the database or within our genotyped families, and thus inter-marker distances were assigned as 0.01cM.

Chromosome 12 haplotypes in the PARK8 region were established for those families in which chromosome phase for mutation-carrying individuals could be deduced, thereby determining which alleles co-segregated with the *LRRK2* G2019S mutation in each family. For those affected individuals in whom the associated allele for a marker could not be determined, both alleles are given.

The age-dependent penetrance was estimated as the probability of a gene carrier becoming affected, at a given age, within the 13 families. The number of affected mutation carriers, for each decade, was divided by the total number of affected individuals, plus the number of unaffected carriers within that range. For some affected family members no DNA was available and only historical data on the disease course was obtained. These individuals were excluded from penetrance calculations.

Results

As mentioned previously, we identified 13 affected probands (i.e. 13 patients) who carry a heterozygous G6055A mutation in exon 41 of the *LRRK2* gene. The mutation leads

to a G2019S amino acid substitution of a highly conserved residue within the predicted activation loop of the MAPKKK (Mitogen-Activated Protein Kinase Kinase Kinase) domain (figure 1). After genotyping a total of 42 additional family members, 22 additional subjects were found to carry the mutation, seven with a diagnosis of PD (shown in table 3 below). One affected member of family P-089 did not carry the mutation and, for the purposes of this study, was considered a phenocopy and excluded from further analyses. Seven families originated from Norway, three were from the United States, two from Ireland, and one was from Poland. One family from the United States descended from Russian/Rumania, and another from Italy. For only one family (family 111), the ethnic origin was unknown. The *LRRK2* G2019S mutation segregates with disease in all kindreds, consistent with autosomal dominant transmission. To ensure patient confidentiality, simplified versions of the family pedigrees are presented in figure 2. There was no evidence of the mutation in the 2260 control samples.

Age at onset of clinical symptoms was quite variable, even within the same family. Family 1120, a family from the United States, had both the earliest and latest age at onset for a patient. The youngest affected subject had an onset at 39 years, whereas the oldest carrier presented with initial symptoms at 78 years. Where recorded, most *LRRK2* G2019S carriers have late-onset disease (>50 years at onset). The mean age at onset of affected mutation carriers was 56.8 years (range 39-78 years, n=19). Unaffected carriers have a mean age of 53.9 years (range 26-74 years, n=14). The penetrance of the mutation was found to be highly age-dependent, increasing from 17% at the age of 50 to 85% at the age of 70 (figure 4).

Table 3

Demographic and Clinical Information for 13 Families with *LRRK2* G2019S

	FINDINGS FOR FAMILY												
CHARACTERISTIC	P-063	P-089	P-104	P-241	P-369	P-394	F05	1210	111	1120	PD66	3211	1P
Country of origin	Norway	Norway	Norway	Norway	Norway	Norway	Norway	United States	United States	United States	Ireland	Ireland	Poland
No. of generations	3	4	3	3	3	4	4	2	2	3	1	2	1
No. of affected individuals	2	4	4	1	3	4	5	2	3	3	1	3	1
No. of typed individuals affected (unaffected)	1 (6)	2 (8)	1 (1)	1 (4)	2 (3)	1 (1)	3 (6)	1 (0)	2(0)	3 (3)	1 (0)	2 (6)	1 (0)
No. of typed generations	2	3	1	2	1	2	2	1	1	2	1	1	1
Age[a] at onset in years (range)	59 (53–65)	59 (43–70)	58	60	50 (43–61)	66	64 (61–70)	65	58 (57–58)	59 (39–78)	41	46 (40–52)	73
Maximum mLOD score	0	.30	0	0	.60	0	.90	0	.09	.30	0	.30	0

[a]Average ages at onset are given when affected individuals. $n \geq 2$

Evidence for linkage (the statistical burden of proof that this mutation causes disease) to the PARK8 locus was found across families, with a combined maximum multipoint LOD score of 2.41 [for all 14 markers], corresponding to a P value of 4,3 x 10^{-4}. As only a defined chromosomal region was investigated, rather than a genome-wide search, this LOD score exceeds that required for significance, P=0.01 (Lander and Kruglyak 1995). A positive LOD score was found in all families where more then one affected subject was genotyped (table 3).

All affected members from the different families, except the individual in family P-089 who did not carry the mutation, appear to share a common haplotype on chromosome 12 the *LRRK2* gene locus (figure 3). Haplotypes can be established with certainty in nine of the families, and all mutation carriers in these families share alleles for four STR markers and 4 single nucleotide polymorphisms (SNPs) in the *LRRK2* gene locus. These markers are *LRRK2* D12S2516, D12S2518, D12S2519, D12S2520 and SNPs rs7966550, rs1427263, rs11176013, rs11564148. For the remaining families, the number of available samples from relatives was not sufficient to determine phase. However, the genotypes in these cases are consistent with a common *LRRK2* G2019S allele. D12S2516 is located in intron 29 and D12S2518 is located in intron 44 of the *LRRK2* gene, whereas the two other shared markers are positioned 3' of the gene. Using the physical position of the shared and non-shared markers, the size of the shared haplotype is between 145 kb and 154 kb.

Discussion

We have identified a novel *LRRK2* mutation, G2019S, which co-segregates with autosomal dominant parkinsonism in 13 kindreds originating from several European populations. Positive LOD scores were obtained in multiplex families, and combined they provide significant support for the PARK8 locus. *LRRK2* G2019S mutation was absent in a large number of control subjects, and of similar ethnicity. The number of families linked to *LRRK2* in this and previous studies now explains the majority of genetically defined autosomal dominant parkinsonism.

The mean age at onset of affected *LRRK2* G2019S carriers was 56.8 years, and comparable to that of patients in other families linked to PARK8 (Funayama et al. 2002; Paisan-Ruiz et al. 2004; Zimprich et al. 2004a). The majority of patients present with late-onset disease, indistinguishable from typical idiopathic PD. Disease penetrance is age-dependent, and increases in a linear fashion from 17% at the age of 50 to 85% at the age of 70. Age is the single most consistent risk factor for development of PD and other neurodegenerative disorders (Lang and Lozano 1998), and an important risk factor in *LRRK2* associated parkinsonism. Interestingly, age at onset was variable in this study, both within and between different families, suggesting other susceptibility factors, environmental or genetic, may influence the phenotype.

Although our findings clearly indicate that *LRRK2* mutations account for a substantial proportion of familial late-onset parkinsonism, historically, cross-sectional twin studies have not supported a genetic etiology for late-onset PD (Tanner et al. 1999; Wirdefeldt et

al. 2004). The age-associated penetrance of *LRRK2* mutations provides some explanation as even large and well designed twin studies are underpowered to detect incompletely penetrant mutations (Simon et al. 2002). *LRRK2* mutations were also found in apparently sporadic PD patients; three of the patients in this study did not have any known affected first- or second-degree relatives. However, a caveat of age-dependent penetrance is that carriers may die of other diseases, before manifesting or being diagnosed with PD. Thus, it seems difficult to separate sporadic and familial PD, or to hypothesize environmental causes to be more important in one group and genetic causes more prominent in the other. In light of these results, a family history of parkinsonism, previously considered an exclusion criterion for a diagnosis of PD, must be reconsidered (Hughes et al. 1992).

LRRK2 is a member of the recently defined ROCO protein family (Bosgraaf and Van Haastert 2003). In human, mouse and rat, members of the ROCO protein family have five conserved domains (figure 1). The kinase domain belongs to the MAPKKK subfamily of kinases. The active sites of all kinases are located in a cleft between an N-terminal and a C-terminal lobe, typically covered by an 'activation loop', in an inactive conformation. The activation loop must undergo crucial structural changes to allow access to peptide substrates and to orientate key catalytic amino acids (Huse and Kuriyan 2002). In different kinases, the activation loop starts and ends with the conserved residues asp-phe-gly (DFG) and ala-pro-glu (APE), respectively (Dibb et al. 2004). Of note, the *LRRK2* G2019S substitution changes a highly conserved amino acid at the start of this loop (figure 5). In a German family we previously described, an I2020T mutation is located in an adjacent codon (Zimprich et al. 2004a). In other kinases, oncogenic mutations in residues within the activation loop of the kinase domain have an activating effect (Davies et al. 2002), thus we postulate *LRRK2* G2019S and I2020T mutations may have an effect on its kinase activity.

The age of an allele may be estimated from the genetic variation among different copies (intra-allelic variation), or from its frequency (Slatkin and Rannala 2000). However, the local recombination rate on chromosome 12q12 is unknown, as is the frequency of the G2019S mutation in the general population. Nevertheless, at centromeres there is generally a dearth in recombination; indeed no crossovers have been observed between *LRRK2* flanking markers D12S2194 and D12S1048 in our studies, or within CEPH families (MAP-O-MAT web site). The physical size of the shared haplotype is also small, between 145 kb and 154 kb, and the allele is widespread in families from several European populations. Hence, the mutation is likely to be ancient and may be relatively common in specific

populations. These data suggest a substantial proportion of late-onset PD will have a genetic basis.

Electronic-Database Information

The physical position of markers is from NCBI build 34. Accession numbers and URLs for data presented herein are as follows:

Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim/
MAP-O-MAT, http://compgen.rutgers.edu/mapomat
RepeatMasker, http://www.repeatmasker.org/

References

Bonifati V, Rizzu P, van Baren MJ, Schaap O, Breedveld GJ, Krieger E, Dekker MC, Squitieri F, Ibanez P, Joosse M, van Dongen JW, Vanacore N, van Swieten JC, Brice A, Meco G, van Duijn CM, Oostra BA, Heutink P (2003) Mutations in the DJ-1 gene associated with autosomal recessive early-onset parkinsonism. Science 299:256-9

Bosgraaf L, Van Haastert PJ (2003) Roc, a Ras/GTPase domain in complex proteins. Biochim Biophys Acta 1643:5-10

Chartier-Harlin MC, Kachergus J, Roumier C, Mouroux V, Douay X, Lincoln S, Levecque C, Larvor L, Andrieux J, Hulihan M, Waucquier N, Defebvre L, Amouyel P, Farrer M, Destee A (2004) Alpha-synuclein locus duplication as a cause of familial Parkinson's disease. Lancet 364:1167-9

Davies H, Bignell GR, Cox C, Stephens P, Edkins S, Clegg S, Teague J, et al. (2002) Mutations of the BRAF gene in human cancer. Nature 417:949-54

de Rijk MC, Breteler MM, Graveland GA, Ott A, Grobbee DE, van der Meche FG, Hofman A (1995) Prevalence of Parkinson's disease in the elderly: the Rotterdam Study. Neurology 45:2143-6

Dibb NJ, Dilworth SM, Mol CD (2004) Switching on kinases: oncogenic activation of BRAF and the PDGFR family. Nat Rev Cancer 4:718-27

Farrer M, Kachergus J, Forno L, Lincoln S, Wang DS, Hulihan M, Maraganore D, Gwinn-Hardy K, Wszolek Z, Dickson D, Langston JW (2004) Comparison of kindreds with parkinsonism and alpha-synuclein genomic multiplications. Ann Neurol 55:174-9

Forno LS (1996) Neuropathology of Parkinson's disease. J Neuropathol Exp Neurol 55:259-72

Funayama M, Hasegawa K, Kowa H, Saito M, Tsuji S, Obata F (2002) A new locus for Parkinson's disease (PARK8) maps to chromosome 12p11.2-q13.1. Ann Neurol 51:296-301

Gelb DJ, Oliver E, Gilman S (1999) Diagnostic criteria for Parkinson disease. Arch Neurol 56:33-9

Hughes AJ, Daniel SE, Kilford L, Lees AJ (1992) Accuracy of clinical diagnosis of idiopathic Parkinson's disease: a clinico-pathological study of 100 cases. J Neurol Neurosurg Psychiatry 55:181-4

Huse M, Kuriyan J (2002) The conformational plasticity of protein kinases. Cell 109:275-82

Kitada T, Asakawa S, Hattori N, Matsumine H, Yamamura Y, Minoshima S, Yokochi M, Mizuno Y, Shimizu N (1998) Mutations in the parkin gene cause autosomal recessive juvenile parkinsonism. Nature 392:605-8

Kong A, Cox NJ (1997) Allele-sharing models: LOD scores and accurate linkage tests. Am J Hum Genet 61:1179-88

Kruger R, Kuhn W, Muller T, Woitalla D, Graeber M, Kosel S, Przuntek H, Epplen JT, Schols L, Riess O (1998) Ala30Pro mutation in the gene encoding alpha-synuclein in Parkinson's disease. Nat Genet 18:106-8

Lander E, Kruglyak L (1995) Genetic dissection of complex traits: guidelines for interpreting and reporting linkage results. Nat Genet 11:241-7

Lang AE, Lozano AM (1998) Parkinson's disease. First of two parts. N Engl J Med 339:1044-53

Mata IF, Lockhart PJ, Farrer MJ (2004) Parkin genetics: one model for Parkinson's disease. Hum Mol Genet 13 Spec No 1:R127-33

Paisan-Ruiz C, Jain S, Evans EW, Gilks WP, Simon J, van der Brug M, de Munain AL, Aparicio S, Gil AM, Khan N, Johnson J, Martinez JR, Nicholl D, Carrera IM, Pena AS, de Silva R, Lees A, Marti-Masso JF, Perez-Tur J, Wood NW, Singleton AB (2004) Cloning of the Gene Containing Mutations that Cause PARK8-Linked Parkinson's Disease. Neuron 44:595-600

Pals P, Lincoln S, Manning J, Heckman M, Skipper L, Hulihan M, Van den Broeck M, De Pooter T, Cras P, Crook J, Van Broeckhoven C, Farrer MJ (2004) alpha-Synuclein promoter confers susceptibility to Parkinson's disease. Ann Neurol 56:591-5

Polymeropoulos MH, Lavedan C, Leroy E, Ide SE, Dehejia A, Dutra A, Pike B, Root H, Rubenstein J, Boyer R, Stenroos ES, Chandrasekharappa S, Athanassiadou A, Papapetropoulos T, Johnson WG, Lazzarini AM, Duvoisin RC, Di Iorio G, Golbe LI, Nussbaum RL (1997) Mutation in the alpha-synuclein gene identified in families with Parkinson's disease. Science 276:2045-7

Simon DK, Lin MT, Pascual-Leone A (2002) "Nature versus nurture" and incompletely penetrant mutations. J Neurol Neurosurg Psychiatry 72:686-9

Singleton AB, Farrer M, Johnson J, Singleton A, Hague S, Kachergus J, Hulihan M, Peuralinna T, Dutra A, Nussbaum R, Lincoln S, Crawley A, Hanson M, Maraganore D, Adler C, Cookson MR, Muenter M, Baptista M, Miller D, Blancato J, Hardy J, Gwinn-Hardy K (2003) alpha-Synuclein locus triplication causes Parkinson's disease. Science 302:841

Slatkin M, Rannala B (2000) Estimating allele age. Annu Rev Genomics Hum Genet 1:225-49

Spillantini MG, Schmidt ML, Lee VM, Trojanowski JQ, Jakes R, Goedert M (1997) Alpha-synuclein in Lewy bodies. Nature 388:839-40

Tanner CM, Ottman R, Goldman SM, Ellenberg J, Chan P, Mayeux R, Langston JW (1999) Parkinson disease in twins: an etiologic study. Jama 281:341-6

Valente EM, Abou-Sleiman PM, Caputo V, Muqit MM, Harvey K, Gispert S, Ali Z, Del Turco D, Bentivoglio AR, Healy DG, Albanese A, Nussbaum R, Gonzalez-Maldonado R, Deller T, Salvi S, Cortelli P, Gilks WP, Latchman DS, Harvey RJ, Dallapiccola B, Auburger G, Wood NW (2004) Hereditary early-onset Parkinson's disease caused by mutations in PINK1. Science 304:1158-60

Vila M, Przedborski S (2004) Genetic clues to the pathogenesis of Parkinson's disease. Nat Med 10 Suppl:S58-62

Wirdefeldt K, Gatz M, Schalling M, Pedersen NL (2004) No evidence for heritability of Parkinson disease in Swedish twins. Neurology 63:305-11

Zarranz JJ, Alegre J, Gomez-Esteban JC, Lezcano E, Ros R, Ampuero I, Vidal L, Hoenicka J, Rodriguez O, Atares B, Llorens V, Gomez Tortosa E, del Ser T, Munoz DG, de Yebenes JG (2004) The new mutation, E46K, of alpha-synuclein causes Parkinson and Lewy body dementia. Ann Neurol 55:164-73

Zimprich A, Biskup S, Leitner P, Lichtner P, Farrer M, Lincoln S, Kachergus J, Hulihan M, Uitti RJ, Calne DB, Stoessl AJ, Pfeiffer RF, Patenge N, Carbajal IC, Vieregge P, Asmus F, Muller-Myhsok B, Dickson DW, Meitinger T, Strom TM, Wszolek ZK, Gasser T

(2004a) Mutations in LRRK2 Cause Autosomal-Dominant Parkinsonism with Pleomorphic Pathology. Neuron 44:601-7

Zimprich A, Muller-Myhsok B, Farrer M, Leitner P, Sharma M, Hulihan M, Lockhart P, Strongosky A, Kachergus J, Calne DB, Stoessl J, Uitti RJ, Pfeiffer RF, Trenkwalder C, Homann N, Ott E, Wenzel K, Asmus F, Hardy J, Wszolek Z, Gasser T (2004b) The PARK8 locus in autosomal dominant parkinsonism: confirmation of linkage and further delineation of the disease-containing interval. Am J Hum Genet 74:11-9

Patent claims

1. A polynucleotide consisting of the base sequence of SEQ ID NO: 2, or a complementary strand thereto, wherein the X is one of the group being defined by the bases A, C or T

2. A polynucleotide according to claim 1, wherein the polynucleotide is at the least a part of a gene.

3. A recombinant vector comprising a polynucleotid according to claim 1.

4. A DNA probe specific for the polynucleotide of claim 1, wherein it contains more than 10 consecutive nucleotides from the nucleotide, or the complementary strand.

5. A method of proving parkinsonism inheritance, by screening a sample of material taken from the subject of interest, with a probe according to claim 5.

6. DNA primer specific for the polynucleotide of claim 1, wherein it contains more than 10 consecutive nucleotides from the nucleotide, or the complementary strand.

7. Use of a polynucleotide according to claim 1, or a vector according to claim 4, to transfect an organism.

8. Use according to claim 7, wherein the organism is a mammal.

9. A peptide consisting of the base sequence of SEQ ID NO:1, wherein the x is not glycine.

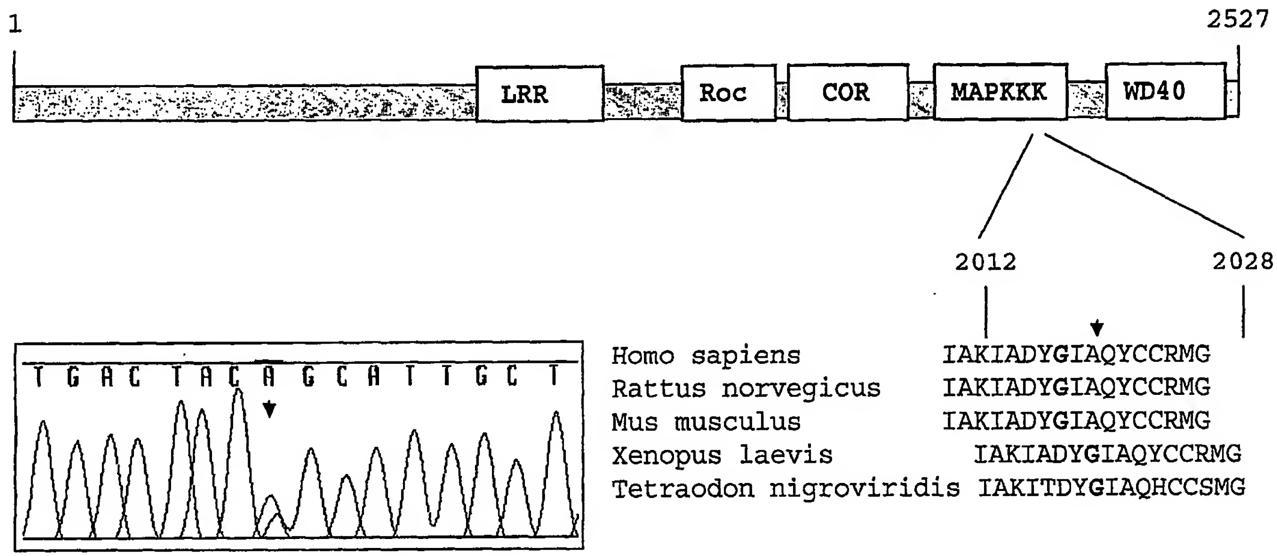
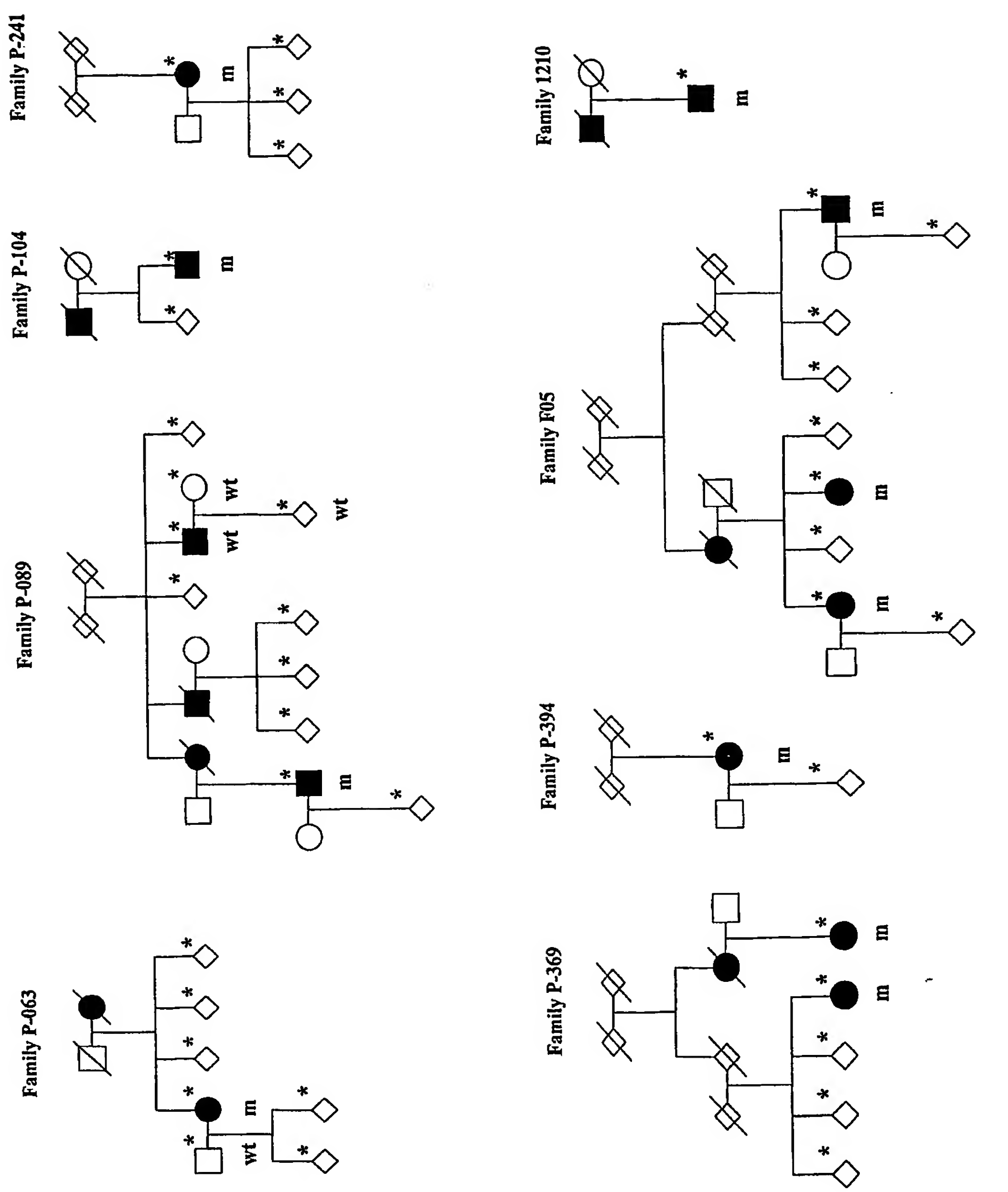


Figure 1. Schematic drawing of *LRRK2* with predicted protein domains

(LRR – leucine rich repeat, Roc – Ras in complex proteins, COR – domain C-terminal of Roc, MAPKKK – mitogen-activated protein kinase kinase kinase, WD40 – WD40 repeats). The human *LRRK2* protein sequence in the region of the G2019S mutation is aligned with orthologs from rat (XP_235581), mouse (AAH34074), frog (AAH76853), and puffer fish (CAG05593). The chromatogram shows the 6055G>A transition (G2019S)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m
Family P-104
m
Family P-089
wt
wt
wt
m
Family P-063
m
wt
Family 1210
m
Family F05
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m
Family P-369
m
m

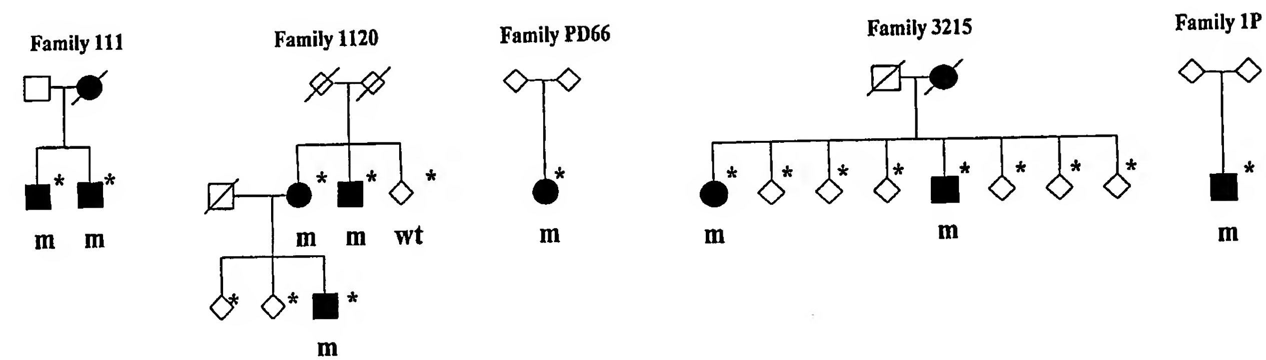


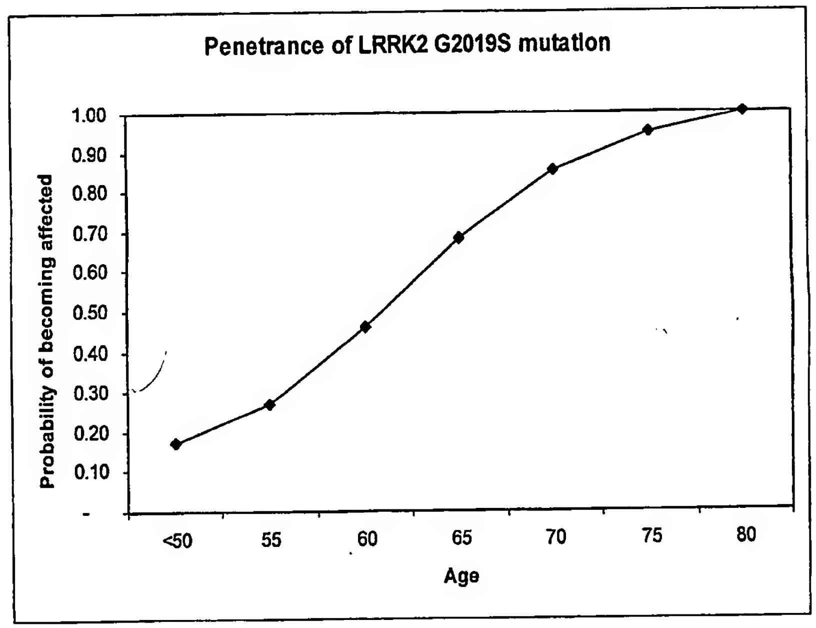
Figure 2. Pedigrees of families with *LRRK2* G2019S
□ and○ denotes sexes, and ◊ denotes that the sex is not given. A diagonal line across the symbol denotes that the person is dead, and thus that he/she has not been tested. Blackened symbols denote affected family members with parkinsonism. An asterisk denotes genotyped individual, with "m" for mutation carriers and "wt" for wild-type *LRRK2*. To protect confidentiality, the genotypes and genders of some unaffected individuals are not shown.

Figure 3. Chromosome 12q12 STR markers on the disease haplotype (PARK8).

	Family proband												
Marker	P-063	P-089	P-104	P-241	P-369	P-394	F05	1210	1120	111	3215	PD66	1P
D12S87	160	160	164	164	-	156	166	156/158	164	160	158	156/166	156/158
D12S1648	120	120	122	122	122	110	110	122/124	110	110	110	120/134	128/130
D12S2080	188	188	188	188	188	188	188	184/192	188	180	184	188/192	184/188
D12S2194	265	265	265	265	265	265	261	253/261	257	257	253	245/249	249/261
-31Kb	290	290	290	290	290	290	290	290/290	290	290	290	290/293	284/290
LRRK2_69Kb	223	223	223	223	223	223	223	219/223	223	223	223	215/215	211/219
LRRK2_84Kb	253	253	253	253	253	253	253	253/253	253	253	253	253/253	253/253
LRRK2_129Kb	151	151	151	151	151	151	151	151/151	151	151	151	151/151	151/151
212Kb	132	132	132	132	132	132	132	132/132	132	132	132/138	132/138	132/134
243Kb	315	315	315	315	315	315	315	315/315	315	315	315/309	315/312	315/300
378Kb	189	189	189	189	189	189	189	189/193	193	193	191	183/189	183/187
D12S1048	214	214	214	214	214	211/214	214	214/223	214	214	223	211/214	211/226
D12S1301	112	116	120	120	116	116	116	108/116	100	120	116	100/116	100/100
D12S1701	95	97	91	91	95	95/97	97	95/101	92	91/95	95	97/101	91/97
Country of origin	Norway							United States			Ireland		Poland

Genotypes for probands from13 families with *LRRK2* G2019S are shown, those shared are highlighted in grey.

Figure 4. Probability of becoming affected by parkinsonism, in *LRRK2*G2019S carriers, as a function of age.



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LRRK2     DYGIAQ----YCCRMGIKTSEGTPGFRAPE
LRRK1     DYGISR----QSFHEGALGVEGTPGYQAPE
MATK      DFGLAK-----AERKGLDSSRLPVKWTAPE
PDGFRA    DFGLARDIMHDSNYVSKGSTFLPVKWMAPE
MAP3K10   DFGLAR----EWHKTTKMSAAGTYAWMAPE
DAPK1     DFGN-----------EFKNIFGTPEFVAPE
BRAF      DFGLATVKSRWSGSHQFEQLSGSILWMAPE
```

Figure 5. Aligned amino acid sequences of the activation loop of different human kinases.

In most kinases, the activation loop starts and ends with the conserved residues DFG and APE, respectively . In *LRRK2* and *LRRK1* phenylalanine is changed to tyrosine, an amino acid with a similar structure. (LRRK2 – leucine-rich repeat kinase 2, LRRK1 – leucine-rich repeat kinase 1, MATK – megakaryocyte-associated tyrosine kinase, PDGFRA – platelet-derived growth factor receptor alpha, MAP3K10 – mitogen-activated protein kinase kinase kinase 10, DAPK1 – death-associated protein kinase 1, BRAF – v-raf murine sarcoma viral oncogene homolog B1)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/NO2005/000465

A. CLASSIFICATION OF SUBJECT MATTER

IPC: see extra sheet
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC: C12Q, C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EPO-INTERNAL, WPI DATA, PAJ, BIOSIS, MEDLINE, EMBASE, SEQUENCE SEARCH (EBI-REGISTRY)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	ZIMPRICH ALEXANDER ET AL, "Mutations in LRRK2 Cause Autosomal-Dominant Parkinsonism with Pleomorphic Pathology", Neuron November 18, 2004, Vol. 44, p. 601-607, page 604, column 2, paragraph 2, figure 3, table 1, abstract --	1-9
P,X	KACHERGUS JENNIFER ET AL, "Identification of a Novel LRRK2 Mutation Linked to Autosomal Dominant Parkinsonism: Evidence of a Common Founder across European Populations", Am.J.Hum.Genet 2005, Vol. 76, p. 672-680 -- --------	1-9

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
18 April 2006	20-04-2006
Name and mailing address of the ISA/ Swedish Patent Office Box 5055, S-102 42 STOCKHOLM Facsimile No. +46 8 666 02 86	Authorized officer Terese Sandström/ELY Telephone No. +46 8 782 25 00

International patent classification (IPC)

C12Q 1/68 (2006.01)
C12N 9/12 (2006.01)

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